# Sensitivity of the Hawaiian Mushroom Coral *Fungia scutaria* to the Pesticide Chlorpyrifos: Cleavage and Early Planula Larva Stages

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## Abstract

The early developmental and larval stages of the solitary coral *Fungia scutaria* were examined for their sensitivity to the pesticide chlorpyrifos (0,0-diethyl 0-(3,5,6,-trichloro-2-pyridyl) phosphoro-thioate). Freshly spawned gametes were mixed together in varying amounts of the pesticide chlorpyrifos and observed for successful fertilization and cleavage. If fertilization and cleavage were successful, then motile larvae were observed 10 h after fertilization. In separate study, 18 h larvae were incubated for 24 h in varying levels of chlorpyrifos. In the fertilization/cleavage studies, mortalility was observed at added chlorpyrifos levels of 10 ppm ($LC_{50} \cong 8.1$ ppm), far above the reported solubility of chlorpyrifos in seawater. The 18 h larvae were more sensitive, exhibiting mortality (as determined by the lack of motility) at 5 ppm ($EC_{50} \cong 1.2$ ppm), still above the reported solubility of chlorpyrifos in seawater. These studies demonstrated that these immature stages exhibit high tolerance for this pesticide.


## Introduction

Corals and coral reef ecosystems are immensely interesting for their beauty, as well as their intrinsic biological and economic values. Reef corals themselves are interesting because of their symbiotic associations with algae and their roles as producers, consumers, competitors for space, habitat providers, reef builders, and modifiers of water chemistry. Coral reef ecosystems have been characterized as being extremely productive and exhibiting high biological productivity (Sargent and Austin 1949; Odum and Odum 1955; Gordon and Kelly 1962; Johannes et al. 1972; Smith and Marsh 1973; Lewis 1977; Kinsey 1977; Erez 1990; Sorokin 1990). In many respects, coral reefs may be regarded as the marine counterparts to tropical rain forests. Reefs also provide resources for human use (Craik et al. 1990), such as food, objects of cultural value, and recreational playgrounds. Finally the reef protects coastlines from the erosional force of waves.

Recently, considerable interest has focused on the contribution of anthropogenic disturbance to the decline in the abundance and diversity of organisms in coral reef ecosystems.

Increased sedimentation, resource exploitation, dredging, temperature stress, sewage effluent, and toxic chemical pollution have been cited as anthropogenic factors threatening coral reefs and their component organisms (Gomez 1988; Rogers 1990; Craik et al. 1990; Grigg and Dollar 1990; Buddemeier 1993; Wilkinson 1993; Richmond 1993).

Among the types of toxic chemical pollution that may impact coral reef environments are pesticides used to control insect pests in both urban and rural watersheds (Olafson 1978; Solbakken et al. 1984; Hallecher et al. 1985; Glynn et al. 1989; Acevedo 1991; Te 1992a; Hunter et al. in prep.). However, despite the recognition that pesticides in non-point source runoff have the potential to damage coral reef ecosystems, little work has been done to assess the impact of pesticides on reef corals themselves (Glynn et al. 1986; Acevedo 1991; Te 1992a).

Chlorpyrifos (0,0-diethyl 0-(3,5,6,-trichloro-2-pyridyl) phosphoro-thioate) is the active ingredient in Dursban®, a common pesticide manufactured by Dow Chemical (Allender and Keegan 1991). Dursban® may be found in many hardware stores and gardening supply centers in many urbanized areas (Racke in press). It is a broad spectrum organophosphate insecticide commonly used against ants, termites, cockroaches, fleas, mosquitos and insect pests of turfgrass and ornamental plants (EPA 1986). As a treatment for mosquito control it has been demonstrated to be detrimental to nontarget species (Hurlbert et al. 1970, 1972; McEwen et al. 1986; Odenkirchen and Eisler 1988).

Domestic use of chlorpyrifos in the United States was about 3.6 million kg in 1982 (EPA 1982). Because chlorpyrifos is not a restricted chemical in Hawaii, there is apparently no federal or state monitoring of chlorpyrifos use in Hawaii. In addition, there is no information available regarding chlorpyrifos levels in the coastal waters of the Hawaiian Islands. However, a recent study investigating the levels of a variety of pesticides in oysters (*Crassostrea gigas*) in Kaneohe Bay, Oahu, did not detect any chlorpyrifos above the sensitivity of the assays used (Hunter et al. in prep.).

The only coral species to have ever been evaluated for chlorpyrifos toxicity thus far was *Pocillopora damicornis* (Acevedo 1991; Te 1992). Adult *Pocillopora damicornis* branches

exhibited an $LC_{50}$ for chlorpyrifos (in the form of Dursban®) of 6 parts per billion (ppb) after 96 hours of exposure (Te 1992a). Thus, the commercial pesticide formulation, Dursban®, was found to be extremely toxic to *Pocillopora damicornis*. Approximately one ten millionth of the manufacturer's recommended level of dilution for the treatment of lawns and gardens was sufficient to kill 50% of the test organisms over four days. The exact mechanism of action of killing by the pesticide on corals was not studied.

Conventional wisdom suggests that the developmental and larval stages of animals are usually more sensitive to acute toxic chemical exposure and other environmental stresses than are the corresponding adult stages (Richmond 1993). Thus the use of these immature stages may be useful organisms in ecotoxicological work. With this idea in mind, a number of investigators have recommended that coral planula larvae be used in experimental toxicity tests and for monitoring environmental stress in reef ecosystems (Stebbing and Brown 1984; Brown and Howard 1985; Brown 1988). However, a number of studies (Edmondson 1946; Coles 1985; Esquivel 1986; Acevedo 1991; Te 1991, 1992b) have suggested that the planula larva stage of the coral *Pocillopora damicornis* is more robust to toxic chemicals and environmental stresses than are the adult corals.

In the only study to date evaluating the sensitivity of coral planula larvae (*Pocillopora damicornis*) to pesticides, chlorpyrifos was found to produce mortality at added activities of 1 ppm and greater (Acevedo 1991). Note that this activity may exceed the solubility of chlorpyrifos in seawater by more than tenfold (Schimmel et al., 1983).

Richmond (1993) suggested that environmental stress may be particularly significant in affecting broadcast spawning corals that participate in synchronized multi-species mass spawning events. If, for example, a mass spawning event occurs when pollution levels are high, an entire cohort may be destroyed. Supporting this view, Richmond (1993) cited his unpublished work showing reduced fertilization success when coral gametes were exposed to stream effluent containing a high sediment load.

The present study sought to evaluate the sensitivity of immature stages (fertilization-

cleavage and early planula stages) of the solitary mushroom coral *Fungia scutaria* to acute exposures to chlorpyrifos. Note that no studies presently exist that have evaluated the sensitivity of the earliest development stages of corals to specific pesticides.

*Fungia scutaria* was chosen for this study for the following reasons: *Fungia scutaria*, in contrast to many corals, exhibits separate sexes and both male and female gametes may be collected without contamination; it is a solitary coral species living free and unattached on the reef surface allowing easy, damage-free collection of living animals; it is a broadcast spawner that predictably releases gametes at dusk 1-4 days following the full moon during the months of summer and fall; fertilization may be accomplished *in vitro*; and development to ciliated larva stage is rapid, normally less than 12 hours after spawning (Krupp 1983). If fertilization is inhibited (e.g. by exposure of the eggs and/or sperm to toxic chemicals) the eggs fail to undergo cleavage and disintegrate several hours after spawning. In addition, *Fungia scutaria* is an important reef flat species in Kaneohe Bay where it seems to thrive largely because of its ability to regenerate and proliferate asexually following freshwater flooding events (Krupp et al. 1993).

## Materials and Methods

### Coral Collection and Maintenance

About 20 specimens of the mushroom coral *Fungia scutaria* (Anthozoa: Scleractinia) were collected from shallow patch reef environments in Kaneohe Bay. This number of corals was needed to ensure an adequate supply of spawning males and females during a single evening's spawning event. These corals were maintained in glass bowls (approx. 6-8" in diameter) within a water table receiving a continuous flow of ambient seawater at the Hawaii Institute of Marine Biology. The corals were shaded from full sunlight using a light mesh shade cloth because direct exposure to full sunlight often leads to partial bleaching of tank-maintained corals. Since many of the corals used in this study have been maintained under similar conditions for several years, the culture conditions appeared to provide a stress-free environment for the corals.

The assays were conducted when the corals spawned 1-4 days following the first full moon of the months of July through October in 1993. *Fungia scutaria* usually spawn between 5 and 7 p.m., therefore the water in the table containing the corals was lowered to just below the height of the bowls by 3:00 p.m. This procedure isolated each individual coral before spawning preventing premature mixing of the gametes. A male was identifiable during spawning because of the cloudy, milky appearance of the released sperm. A female was identified by the release of small white eggs (approx. 90 *um* in diameter) appearing as suspended particulate matter.

Gamete Collection and General Fertilization Procedures

The spawned gametes from each individual were collected with a large polyethylene baster and slowly dispensed into 500 mL glass beakers containing about 100 mL filtered seawater (0.22 *um*, Millipore Corp.). The collected gametes from at least three females during each spawning event were pooled. Similarly the sperm from at least three males were pooled. The sperm suspension was used without further dilution. Egg densities were adjusted to about 50 eggs/mL. Higher egg densities often led to embryo fusion and difficulties in counting viable embryos.

For the experimental fertilizations (see below), gametes (1 mL of each gamete suspension) were dispensed slowly using an Eppendorf Repeater Pipette into borosilicate scintillation vials (20 mL capacity, Fisher Scientific) containing 3 mL filtered seawater that was aerated by vigorous shaking prior to use. Thus the final volume in each vial was always 5 mL. After addition of gametes, the vials were sealed and floated cap-side-up in an outdoor seawater table receiving a continuous flow of seawater at the ambient temperature (26-28°C). Aeration in the table provided gentle agitation to the vials.

Fertilization Success at Various Times Following Spawning

A preliminary study was undertaken to determine how long after spawning the eggs and sperm remain viable for experimental fertilization studies. To each vial containing 3 mL filtered seawater, 1 mL of egg suspension (about 50 eggs/mL) was added. At various time intervals following the onset of spawning 1 mL aliquots of the collected sperm suspension (full

strength) were added to these vials (five replicates per time interval). Four separate time series experiments were conducted. Fertilization success was assayed as discussed below.

Chlorpyrifos Sensitivity of Fertilization and Cleavage Stages

For these studies, pure chlorpyrifos (provided by the Evironmental Toxicology unit of the Department of Agricultural Biochemistry, University of Hawaii at Manoa) was used. The stock solution of chlorpyrifos was first dissolved in a methanol carrier (approx. 1 mg/mL) and aliquoted to the incubation vials as described below.

Appropriate volumes of the chlorpyrifos-methanol solution were added to clean, dry vials. After the addition of chlorpyrifos-methanol to the vials, the methanol solvent was allowed to evaporate before the addition of 3 mL filtered seawater to each of the vials. This seawater was added to the vials about 2 h prior to the experimental fertilizations. Final test concentrations (final volume in a vial = 5 mL) volumes after addition of sperm and egg suspensions (approx. 50 eggs/mL) ranged from 10 to 0.01 ppm (five replicates, i.e. vials, per chlorpyrifos concentration tested). Three controls were also conducted: (1) a 250 *u*L methanol control (representing the maximum volume of methanol added to any of the vials; note that as in the experimentals the methanol was allowed to evaporate), (2) a 50 *u*L methanol control (representing an intermediate volume of methanol added to any of the vials; note that as in the experimentals the methanol was allowed to evaporate), and (3) a seawater-only control. Since no quantitative dose-related response due to the methanol could be demonstrated, all control values were treated the same and referred to simply as the "controls". The sperm suspension (1 mL) was added to each vial and preincubated 10 minutes in chlorpyrifos before the addition of 1 mL of egg suspension. A total of four experimental series were conducted.

Quantification of Fertilization Success

*Fungia scutaria* exhibits a rapid rate of development, reaching the first cleavage within 2-4 hours after fertilization (Krupp 1983). Motile ciliated larvae, lacking mouths, are apparent within 10-12 h. It was these ciliated larvae (number of living embryos) that were counted as our measure of fertilization success. Since unfertilized eggs in the scintillation vials

disintegrate within 6-8 h, no unfertilized eggs were observable during these counts.

For the both the time series experiments and the chlorpyrifos tests on coral eggs, fertilization success was calulated as percent fertilization, comparing the number of viable 10 hour embryos to the average starting density of the eggs introduced into each vial as follows:

% fertilization = 100% * [(no. viable embryos per vial)/(average starting egg density)].

Note that for the time series studies, the replicates (five vials for each experiment) were pooled across different time series experiments conducted at different times. While, four separate time series experiments were conducted, not all of these involved the same corresponding time intervals following spawning. Thus some time intervals involve the results of only five vials, while others involve results from 20 vials.

Similarly, the results for the chlorpyrifos tests (five replicates per chlorpyrifos concentration in each of four experiments conducted) were pooled across the experimental series. Thus, each percent fertilization average and standard deviation was based upon the pooled results from these four experimental cross series (n = 20 vials for each concentration tested).

Chlorpyrifos Effects on Planula Larvae

About 50 mL of undiluted sperm suspension were added to a suspension of eggs in about 300 mL seawater shortly after spawning. The next day (approx. 18 h following spawning) motile planula larvae were collected and gently strained through plankton net material (40 *um*). After several washes in filtered seawater, the larval suspension was diluted to yield a density of approximately 50 larvae/mL. One mL aliquots of this final larval suspension were added to 4 mL of filtered seawater in borosilicate vials prepared with or without chlorpyrifos as described above for the fertilization studies. The vials were incubated for 24 h in seawater tables at ambient temperature as described above.

After the 24 h incubation period the larvae were examined and counted. Larvae were

considered alive if they were actively moving. Immobile larvae that could not be stimulated to move were considered to be dead. In some cases, but not all, the dead larvae disintegrated before counting. Percent survivorship was calculated as the number of living larvae relative to the average density of larvae placed in the vials as follows:

% survivorship = 100% * [(no. motile larvae per vial)/(average starting larva density)].

Two separate trials, each involving five replicates, were conducted in September and October 1993. The results from these separate experiments were pooled together. Thus the sample size (n) for each chlorpyrifos concentrations was 10.

Actual Chlorpyrifos Concentrations in the Vials

To determine the realized chlorpyrifos concentrations in the vials (as opposed to added amounts), a set of vials containing the corresponding amounts of chlorpyrifos were prepared without the addition of sperm and eggs (but with final volumes adjusted to 5 mL). These vials were incubated in a constant temperature (27$^{o}$C) water bath for 10 hours. The contents of each of these vials were then passed through PrepSep$^{TM}$-C18 disposable extraction columns (Fisher Scientific P-453) for solid phase extraction of chlorpyrifos from the seawater. After a wash step, the chlorpyrifos was eluted from the columns using acetone. These final acetone elutions (5 mL total volume) were chromatographically assayed for chlorpyrifos content by the Evironmental Toxicology unit of the U.H.M. Department of Agricultural Biochemistry.

## Results

Fertilization Success at Various Times Following Spawning

While there was high variability exhibited among replicates within an experiment and among separate time series experiments, the percentage of live embryos surviving after fertilization declined ($R^2$ = 0.287; linear regression analysis on untransformed values: $F_{1,113}$ = 45.541, $p < 0.001$) as the time of fertilization after the initation of spawning was delayed (Fig. 1). If fertilization did not take place within about 6 hours of spawning, then the eggs did

not develop when exposed to sperm. Note that all no-sperm controls run simultaneous with these trials failed to yield any evidence of successful fertilization and development.

Sensitivity of Developing Embryos to Chlorpyrifos Exposure

Similarly, the chlorpyrifos tests exhibited high variability among replicates and among separate experiments. The controls and lower chlorpyrifos (≤ 1 ppm) treatments yielded average fertilization rates of approximately 70% (Fig. 2). An analysis of variance was conducted on variance-stabilized transformed values (arcsin(√(proportion)), per Sokal and Rohlf, 1981) to detect significant differences among chlorpyrifos treatments and controls. Note that since a few vials exhibited percent fertilization values that exceeded 100% (an artifact of the fact that percent fertilization values were calculated from the average starting density of the eggs during an experimental run), the values were calculated as proportions of the maximum percent fertilization value observed before applying the variance-stabilizing transformation. Because this analysis of variance detected significant differences among the groups ($F_{7,192}$ = 6.631, $p < 0.001$), an *a posteriori* test (Scheffe's F-test) was conducted to detect which groups differed from each other as well as the controls. Only the 10 ppm treatment exhibited a significantly lower ($p < 0.05$) fertilization success when compared to the controls and the other treatments.

To estimate the $LC_{50}$, a sigmoidal logistic-type curve was fit to the raw data using the iterative nonlinear curve-fitting procedure in *DeltaGraph ® Pro* 3.1 on a Macintosh IIvx computer. The form of this equation was

$$Y = \frac{(a - d)}{\left[1 + d + \left(\frac{X}{c}\right)^{b}\right]},$$

where a, b, c, and d were parameters to be estimated. After combining constants, the equation generated ($R^2$ = 0.180) was

$$Y = \frac{73.56}{\left[1.131 + \left(\frac{X}{7.761}\right)^{3.071}\right]},$$

yeilding an $LC_{50}$ estimate of 8.1 ppm. This corresponded well to the $LC_{50}$ of 8.5 ppm estimated through graphical procedures (modified probit method using experimental averages rather than raw data values).

Sensitivity of Planula Larvae to Chlorpyrifos Exposure

Planula larvae were slightly more sensitive to chlorpyrifos exposure (24 h) than were the fertilization/cleavage stages which involved a 10 hour exposure (Fig. 3). Survivorship, as determined by activity level, was high, ranging between 75 and 97% through 0.5 ppm, and falling to zero at 5 ppm. An analysis of variance similar to that described for the percent fertilization experiments was conducted, but with one difference: since the percent survivorship values for 5 and 10 ppm chlorpyrifos treatments were all zero, thus yielding no within-treatment variance estimates, these results were not included. This analysis of variance indicated significant differences among the treatments and controls ($F_{5,74} = 3.596$, $p < 0.01$). An *a posteriori* analysis (Scheffe's F-test) indicated that none of the chlorpyrifos treatments (from 0.01 to 1.0 ppm) differed significantly from the controls. The significant analysis of variance appears to have resulted solely from the difference between the 0.01 chlorpyrifos treatment and the 1.0 ppm treatment ($p < 0.05$).

Using the nonlinear curve-fitting procedures described above, the relationship between percent active larvae and chlorpyrifos content was

$$Y = \frac{80.56}{\left[0.968 + \left(\frac{X}{1.202}\right)^{6.901}\right]},$$

($R^2 = 0.849$) yielding an $EC_{50}$ estimate of 1.2 ppm. This value corresponded reasonably well to an $EC_{50}$ value of 2 ppm estimated using a graphical procedure on probit paper.

Realized Chlorpyrifos Concentrations

Chlopyrifos levels in vials incubated under the same conditions as the developing eggs

demonstrated that the actual concentrations of chlorpyrifos in seawater deviated greatly from the concentrations expected from the added activities when the concentrations were above about 0.1 ppm. NOTE TO READER: this analysis is going to be redone.

## Discussion and Conclusions

The timing of fertilization may have a significant influence on the outcome of studies of this kind. The rapid decline in percent fertilization with time demonstrates the need to conduct fertilizations in a timely manner. Some of the variability observed in the experimental fertilizations conducted might have originated in the time interval involved in carrying out a fertilization series from start to finish. The implication this observation has on fertilization success in the field is that the gametes may quickly loose capacity for fertilization following spawning.

The chlorpyrifos studies with both the gametes and the larvae indicated high tolerance for this pesticide. In fact, it appears as though the added activity must greatly exceed the solubility of chlorpyrifos in seawater in order to observe an effect, thus calculation of $LC_{50}$'s, although done, were not really meaningful in this study. Mortality may have been due to factors other than chemical toxicity, for example, mechanical disruption of gametes or developing larvae due to contact with undissolved slicks of chlorpyrifos in the vials.

The observations made here were consistent with the studies of Acevedo (1991) who used planula larvae from the brooding colonial species *Pocillopora damicornis*. However, adult colonials of *Pocillopora damcornis* may be much more sensitive to chlorpyrifos (Te 1992a). The difference in sensitivity to chlorpyrifos between larval and adult *Pocillopora damicornis* may be due, in part, to procedural differences in the assays. Acevedo (1991), as in the present study, exposed specimens to seawater with pure chlorpyrifos added. Te (1992a), on the other hand, used dilutions of the commercial preparation Dursban® rather than pure solutions of chlorpyrifos in seawater. This commercial preparation undoubtedly contains inert ingredients that function as dispersants and binders which may influence the toxicity of chlorpyrifos or may themselves be toxic to corals.

Another possible explanation involves the mechanism of action of chlorpyrifos on living organisms. Chlorpyrifos may interfer with synapse transmission by inhibiting cholinesterase activity (reference needed). Whether or not a similar mechanism is involved in killing adult corals is not certain. However, fertilization and cleavage, which obviously do not depend upon nervous system function, do not seem to be impacted by chlorpyrifos. Similarly, the larval stages, which may not depend significantly on nerve cell functioning, may also be relatively tolerant to chlorpyrifos.

A number of studies have begun to paint a picture of coral developmental and larval stages being tolerant to a wide range of environmental factors assumed to be stressful to corals (Edmondson 1946; Coles 1985; Esquivel 1986; Acevedo 1991; Te 1991, 1992b; this study). Thus the conventional wisdom that these immature stages are delicate and highly sensitive to exposures to various kinds of environmental pollution may not be valid.

However, given the present level of understanding, this conclusion should not justify a lack of concern for chemical pollution in the coral reef environment. Only few coral species have been subjected to this type of testing and many other chemical pollutants remain to be tested. The interactive effects of other physical and chemical factors need to be explored. Acute and/or chronic exposures to chemical pollutants may affect coral reproduction through other mechanisms, such as gametogenesis and fecundity. In addition, while larval development and survivorship may be robust to chemical toxicants, larval settlement and postsettlement survivorship may be impacted significantly (Hodgson 1990; Te 1992b; Richmond 1993). Finally, as Te (1992a) has shown, adult corals themselves may be highly sensitive to specific chemical toxicants.

Clearly, much more work needs to be done to elucidate the impact of pesticides and other chemical toxicants upon scleractinian corals. This study represents the first successful attempt to investigate the effects of a pesticide on the fertilization and cleavage of eggs from scleractinian corals. In the case of chlorpyrifos, the gametes and planulae appear to be unexpectedly tolerant. The techniques developed here may be applied to investigate the effects of

many other suspected chemical toxicants on corals.

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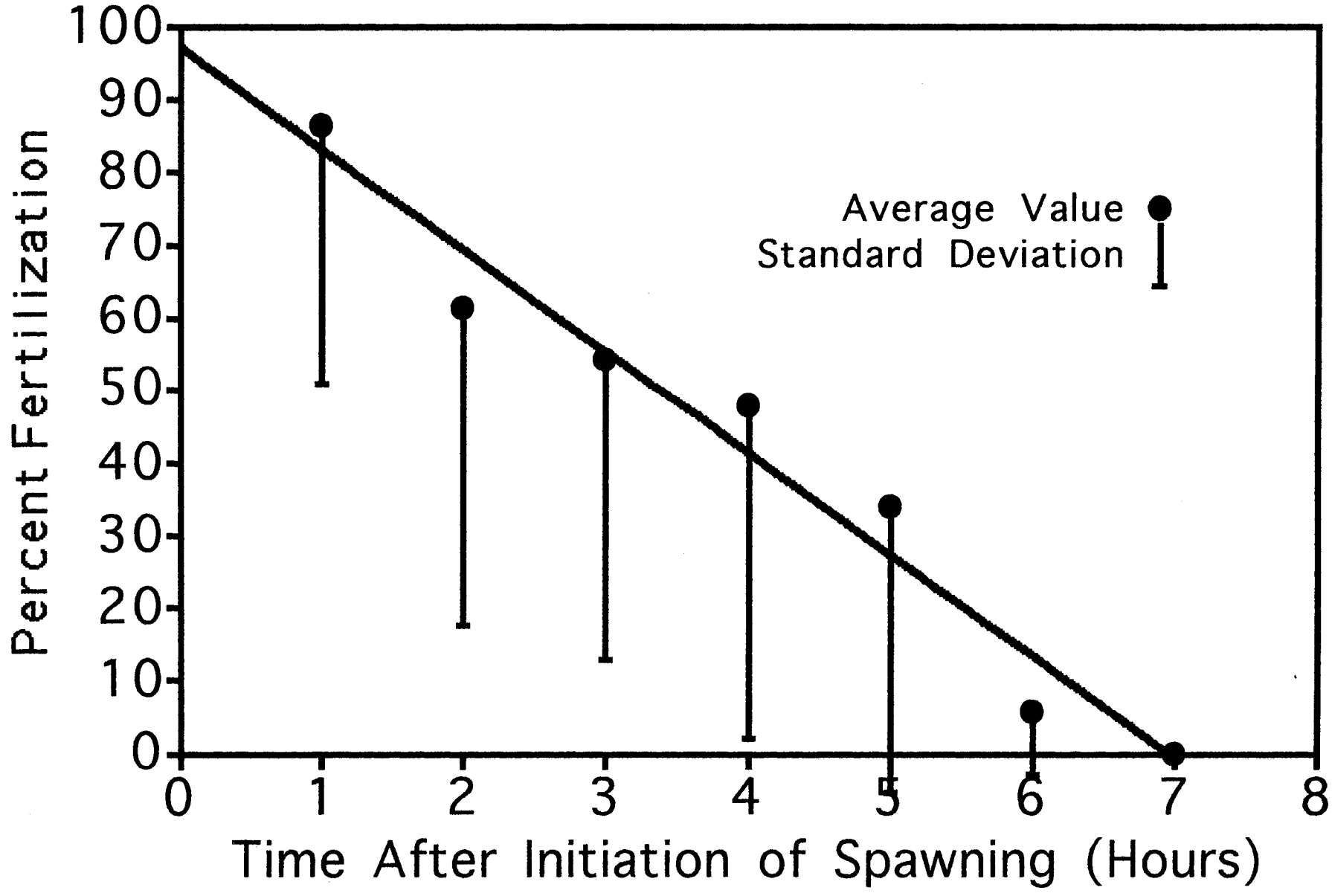
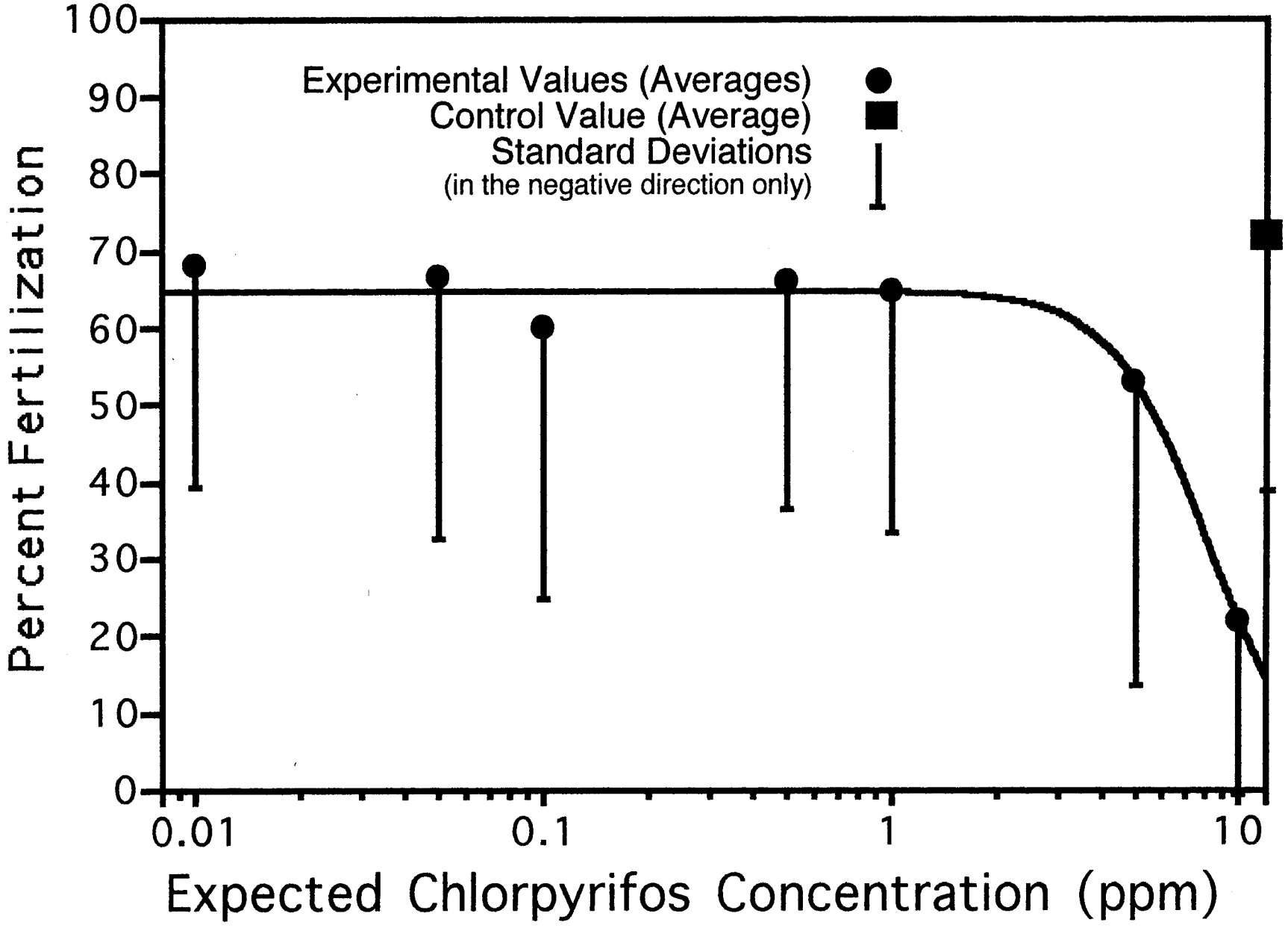
LIST OF FIGURES


100
90
80
70
60
50
40
30
20
10
0
Percent Fertilization
Average Value
Standard Deviation
0
1
2
3
4
5
6
7
8
Time After Initiation of Spawning (Hours)


Experimental Values (Averages)
Control Value (Average)
Standard Deviations
(in the negative direction only)
Percent Fertilization
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
Expected Chlorpyrifos Concentration (ppm)